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Effect of Low Intensity Monochromatic Light Therapy (890 nm) on a Radiation-Impaired, Wound-Healing Model in Murine Skin

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Background and Objective: **The use of low intensity laser and monochromatic light diodes as a therapeutic modality has become popular in a variety of clinical applications, including the promotion of wound repair. Despite this, the clinical evidence base for such application remains sparse; in contrast, recent studies have demonstrated a number of quantifiable photobiological effects associated with such therapy. In the present study, the effect of low intensity monochromatic light irradiation (MLI) at various radiant exposures upon a radiation-impaired wound model in murine skin was investigated.**
Study Design/Materials and Methods: **Male Balb/c mice (n = 50; age matched at 10 weeks) were randomly allocated to five experimental groups (n = 10 each group). In Group 1, mice were left untreated; in Groups 2–5, a well-defined area on the dorsum was exposed to 20 Gy X-ray irradiation. At 72 hours post-irradiation, all mice were anaesthetised and a 7-mm-square area wound was made on the dorsum. All wounds were videotaped alongside a marker scale until closure was complete. In Groups 3–5, mice were treated with MLI (0.18, 0.54, and 1.45 J/cm^2, respectively) three times weekly using a GaAlAs 890 nm multidiode (n = 60) array unit (270 Hz; maximum rated output, 300 mW; Anodyne, Denver, CO). Subsequently, the area of each wound was measured from video using an image analysis system (Fenestra 2.1), and results were analysed using repeated measure and one-factor ANOVA statistical tests.**
Results: **X-ray irradiation caused a significant delay (P = 0.0122) in healing by day 7. MLI at 0.18 J/cm^2 and 0.54 J/cm^2 had no effect upon the rate of wound closure. However, a highly significant (P = 0.0001) inhibition occurred following MLI irradiation at 1.45 J/cm^2 by day 16.**
Conclusion: **These findings provide little evidence of the putative stimulatory effects of monochromatic light irradiation in**

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vivo, but, rather, reveal the potential for an inhibitory effect at higher radiant exposures. Lasers Surg. Med. 23:291–298, 1998.

INTRODUCTION

Photobiostimulation, based on the use of low intensity laser therapy (LILT), or monochromatic light therapy (MLI), has been widely promoted and routinely used for over two decades in a number of centres in Europe and Asia for the treatment of a variety of conditions, based upon claimed effects that include the promotion of wound healing, reduction of oedema, and the relief of pain of various aetiologies [e.g., 1–4]. However, despite such wide clinical usage and the plethora of research in the area, the application of this modality has always provoked scepticism [5]. This is mainly due to the lack of any clear mechanism of action, coupled with the poor quality of many of the published reports in the area [3]. Furthermore, the wide range of irradiation parameters employed by various authors confounds attempts to compare conflicting findings.

Perhaps the most convincing evidence for the claimed clinical benefits of low intensity laser and monochromatic light therapy is derived from laboratory studies on isolated cell lines and (to a lesser degree) animal studies. At the cellular level, claimed photobiostimulatory effects have included activation of fibroblasts [6–8], enhanced neovascularisation [6,7], increased collagen synthesis [9,10], an enhanced mitochondrial electron transport system [8], and stimulation of macrophage activity [11,12]. It would thus appear that these devices do potentially induce measurable biological effects on certain cell types [13–17]. However, although these in vitro studies are of value in determining a potential mechanism of action of these modalities, such research cannot replicate the complex process occurring during wound healing within the intact animal. Thus such in vitro cultures represent an incomplete approach for the study of wound dynamics. For this reason, in vivo research is essential to evaluate the therapeutic effectiveness of novel modalities [18].

In vivo research to date has utilized a variety of animal models to investigate the effects of this modality. In addition, such studies have employed a wide range of irradiation parameters.

Most wound healing studies on animals have investigated the effects of He-Ne laser irradiation, the most popularly used devices both experimentally and (until recently) clinically, using radiant exposures of up to 5 J/cm^2. Kana et al. [19] and Bisht et al. [20] reported accelerated wound closure in rats at a radiant exposure of 4 J/cm^2. Another early study by Surinchak et al. [21] also showed a significant increase in breaking strength of rat skin incisions following irradiation at a slightly lower radiant exposure of 2.2 J/cm^2; interestingly, this group also investigated the effects at both higher and lower dosages (ranging up to 4.5 J/cm^2) and found no stimulatory effects at any other dose. Similar success at 2.41 J/cm^2 was achieved by Takahashi et al. [6], who found a significant increase in neovascularisation of murine tracheal grafts following irradiation at these parameters.

However, not all reports to date have been positive. A recent study by Broadley et al. [22] reported no beneficial effects on tensile strength of incisional wounds following irradiation at radiant exposures of between 0.47 J/cm^2 and 1.73 J/cm^2. However, this study only compiled data on day 15 postwounding, so the effect of irradiation earlier in the proliferative stage of wound healing was possibly ignored [23,24]. Atabey et al. [7] investigated the effects of He-Ne laser irradiation on wound healing in rabbits using daily treatments at a radiant exposure of 3.8 J/cm^2 until complete wound closure. Again, the authors reported no stimulatory effect on contraction rates at the parameters investigated. Similarly, Allendorf et al. [25] demonstrated no beneficial effect on wound healing in rats at radiant exposures of between 1–4 J/cm^2. These authors used two wound models to test this modality, a full thickness excisional skin defect and a single longitudinal full thickness skin incision, with no benefit reported for either.

Although it might be reasonably proposed that such variable results may be due to the employment of (perhaps) inappropriately low energy densities, studies investigating the effects of He-Ne laser irradiation upon wound healing employing relatively higher radiant exposures have also reported conflicting results. Rochkind et al. [26] observed accelerated healing and long-lasting systematic effects using a continuous wave He-Ne laser at radiant exposures of 7.6 J/cm^2 and 10 J/cm^2 on open wounds in mice. In contrast, an

earlier study by Kana et al. [9] demonstrated no such stimulatory effects at radiant exposures of 10 J/cm^2 and 20 J/cm^2; interestingly, the higher radiant exposure of 20 J/cm^2 appeared to inhibit healing in rats.

A number of studies have attempted to compare the relative effects of several wavelengths upon wound healing in rodents. Kameya et al. [27] reported that daily irradiation at wavelengths of 632.8 nm, 680 nm, and 830 nm all produced statistically significant reductions in the area of the skin defect when compared to controls. However, although this study reported a greater rate of wound healing (at unspecified dosages), including a more rapid regeneration of capillaries and greater proliferation of connective tissue at all wavelengths investigated, the He-Ne (632.8 nm) and the GaAlAs (680 nm) lasers (i.e., the "red" lasers) were more effective than the GaAlAs (830 nm) laser. Similarly, Al-Watban and Zhang [4] reported stimulatory effects at a variety of radiant exposures ranging between 1–60 J/cm^2 using a variety of wavelengths, but with superior effects using the He-Ne laser (632.8 nm) at a radiant exposure of 20 J/cm^2 compared to (infrared) GaAlAs lasers at 780 nm and 830 nm.

Conflicting results also have been reported using GaAs laser irradiation. Zarkovic et al. [28] reported a significant increase in the rate of wound closure in mice using a GaAs laser at a wavelength of 905 nm daily. The authors indicated that although the systemic effect of laser irradiation was uncertain, this modality could modify the rate of wound healing. A more recent study by Hall et al. [24] investigated the stimulatory effect, again using a GaAs laser (904 nm), in healing skin wounds in rats. However, no beneficial effects were observed over the range of radiant exposures investigated (0.4–4.0 J/cm^2). Unfortunately, however, most of the laboratory-based research to date has been anecdotal, diverse, and singular, which makes comparison of the studies virtually impossible [8,18] and adds to the scepticism that continues to surround this area.

The effectiveness of LILT has been reported in the treatment of crural ulcers [29], soft tissue lesions, and both acute and chronic wound healing [30]. However, details in the reports of such positive clinical effects are insufficient, and the research design is variable (e.g., studies are uncontrolled; see Conlan et al. [8]). Thus positive reports from works based on uncontrolled trials and case studies may indicate nothing more than a placebo effect [24,31–34]. Therefore, to determine the effectiveness of MLI on chronic wounds normally encountered in the clinical setting, a series of well-controlled experiments using a "chronic" wound in animals would perhaps allow rigorous scientific evaluation of this modality. It has been established that infrared laser and monochromatic light sources are now the most commonly used types of laser in clinical practice [2,30] and are used at a range of radiant exposures from 0–15 J/cm^2, [1,35]. Therefore, investigation of the optimum dose of LILT/MLI for stimulating the wound healing process would be a great advantage clinically, as to date, it would appear that no clear picture has emerged as to the best treatment protocol for use in the promotion of "acute" wound healing. From the literature, it is clear that the majority of the studies to date have used young healthy animals. However, a number of authors, e.g., Karu et al. [36], have suggested that the effect of laser irradiation depends on the physiological condition of the organism being treated, as the response observed using normal functional cells and tissues is much weaker (if present at all) than the response observed in tissues displaying pathological conditions or aging. In agreement, many other authors [7,18,22,37] advocate the experimental investigation of LILT and MLI on wounds that exhibit impaired or problematic healing and suggest that these are more compatible to wounds found clinically in elderly and diabetic patients. Such an impaired wound healing model can be induced through inoculation of a bacterium or virus into the wound, the creation of a diabetic state [38] or by X-ray radiation [38–40].

The aim of the current study was to investigate the effect of monochromatic light irradiation (890 nm) upon the rate of wound closure of a radiation-impaired wound in murine skin and, additionally, to determine if any such effect was dose dependent.

MATERIALS AND METHODS

The current investigation, which was approved by the University of Ulster's Ethical committee, used male Balb/c mice, aged-matched at 10 weeks old (n = 50; mean weight = 26.72 g). Animals were supplied with food and water ad libitum and housed individually to prevent them from tampering with each other's wounds. Animals were then randomly assigned to one of five experimental groups (n = 10 each group); Group 1, non-X-ray irradiated control group; Group 2,

X-ray irradiated control group; Group 3, MLI at 0.18 J/cm^2; Group 4, MLI at 0.54 J/cm^2; Group 5, MLI at 1.45 J/cm^2.

The hair on the dorsum of all mice was shaved. Animals in Groups 2–5 were placed individually into custommade lead jigs, which allowed a 4 cm^2 area of dorsal skin to be exposed. This area was marked with indelible ink and the mice exposed to 20 Gy X-ray irradiation using a Siemens Stabillipan X-ray machine (Seimens Ag. Medical Group, Erlagen, Germany). Seventy-two hours following X-ray irradiation, hair on the dorsal surface was re-shaved (where necessary) and the skin cleaned with 70% alcohol. Mice in all groups were then anaesthetised by inhalation using Isofluorane anaesthetic (Abbot Laboratories, Essex, UK) and a 7 × 7 mm area of skin was removed from within the area previously exposed to X-rays. An equivalent area was removed from all mice in Group 1.

In Groups 3–5, mice were irradiated at radiant exposures of 0.18 J/cm^2, 0.54 J/cm^2, and 1.45 J/cm^2, respectively; this was delivered using a pulsed wave, multidiode (GaAlAs) array (Denver, CO) three times per week. The physical parameters of this unit were measured as: wavelength, 890 nm; maximum rated power output, 300 mW (n = 60 diodes); area of irradiation, 22.5 cm^2; pulse frequency, 270 Hz. The unit was calibrated at the beginning of each day. Irradiation times for radiant exposures of 0.18 J/cm^2, 0.54 J/cm^2, and 1.45 J/cm^2 were calculated using the equation:

$$\text{Time (seconds)} = \frac{\text{Energy density (J/cm}^2\text{)}}{\text{Irradiance (W/cm}^2\text{)}}.$$

During irradiation, the flexible pad of the unit was held around the caudal area of the mice just in contact with the dorsal surface, covering the wound site. All animals were anaesthetised by inhalation of Isofluorane anaesthetic before irradiation. Treatment was given three times weekly until complete wound closure. Control groups 1 and 2 received no irradiation.

All wounds were videotaped alongside a marker scale on the day of wounding and three times weekly thereafter until closure was complete. Wound areas were then calculated using an image analysis system (Fenestra 2.1). To allow for variation between initial values, all wound area data were calculated as the fractional change in wound area for each mouse.

Statistical analysis was performed on an Apple Macintosh Performa 6320 computer using Statview 512+; repeated measures and one-factor ANOVA was used, together with posthoc Fisher tests where appropriate.

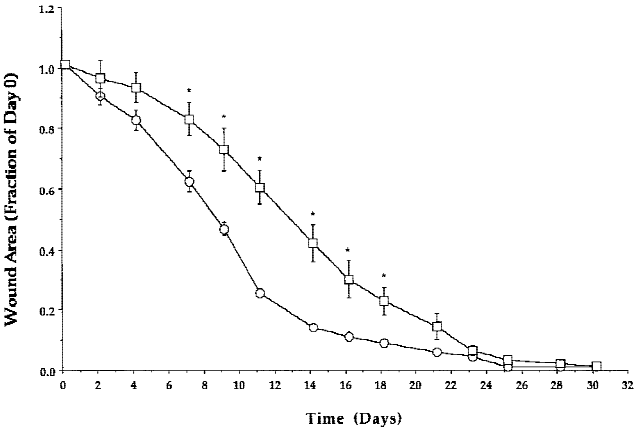

Fig. 1. Wound closure as a fraction of Day 0 for non-X-ray Irradiated Control and X-ray Irradiated Control (points represent means ± s.e.m.; n = 10 for all groups). ○, Group 1, non-X-ray irradiated control; □, Group 2, X-ray irradiated control; *, Statistically significant differences between Group 1 and Group 2 where $P \leq 0.0122$.

RESULTS

Figure 1 shows wound closure (i.e., areas expressed as a fraction of day 0 values) for Groups 1 and 2, the non-X-ray irradiated Control and the X-ray irradiated Control groups, respectively. Whereas both groups follow a similar pattern of wound closure, Group 2 (X-ray irradiated Control group) showed a delay in wound closure that was statistically significant ($P = 0.0122$) when compared to Group 1 (non-X-ray irradiated Control group) by day 7 postwounding. This statistically significant difference continued until day 18. After day 18, the effects of X-ray irradiation upon wound closure were statistically nonsignificant. However, the wounds in Group 1 reached complete wound closure by day 25, whereas the wounds in Group 2 only reached complete wound closure on day 30 postwounding.

Figure 2 shows wound closure as a fraction of Day 0 for Groups 2–5, i.e., the X-ray irradiated Control and the three "treatment" groups. A similar rate of wound closure was observed for all groups; all achieved complete wound closure between days 28 and 30 postwounding. However, one-factor ANOVA showed that whereas wound closure following MLI irradiation at 0.18 and 0.54 J/cm^2 did not differ significantly from the radia-

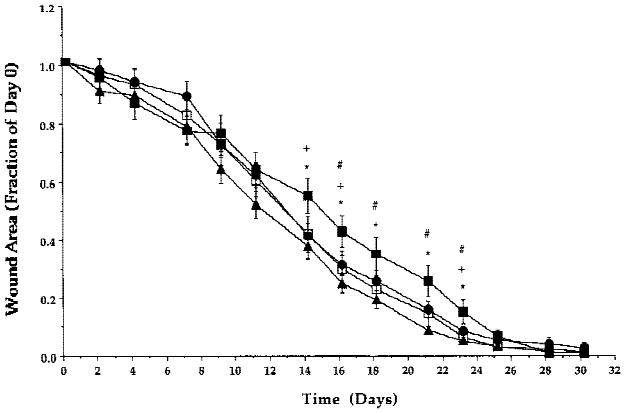

Fig. 2. Wound closure as a fraction of Day 0 for X-ray Irradiated Control and LILT Treatment Groups at 0.5 J/cm^2, 1.5 J/cm^2, and 4 J/cm^2 □, Group 2, X-ray irradiated control; ●, Group 3, X-ray irradiated + MLI 0.18 J/cm^2; ▲, Group 4, X-ray irradiated + MLI 0.54 J/cm^2; ■, Group 5, X-ray irradiated + MLI 1.45 J/cm^2; ≠, Statistically significant differences between Group 2 and Group 5 where $P \leq 0.0205$; +, Statistically significant differences between Group 3 and Group 5 where $P \leq 0.0205$; *, Statistically significant differences between Group 4 and Group 5 where $P \leq 0.0205$.

tion-impaired Control group at any stage of the wound healing process, significant differences were found between the 1.45 J/cm^2 treatment group when compared to the other experimental groups. Statistically significant differences, indicating a (further) delay in the wound healing process, were observed between the 1.45 J/cm^2 laser group and the Control group by day 16 ($P = 0.0001$), and this delay lasted until day 23 postwounding. The 0.18 J/cm^2 laser treatment group differed significantly ($P \leq 0.0205$) from the 1.45 J/cm^2 group at days 14, 16, and 23 postwounding, again demonstrating a delay in the higher radiant exposure group. The group receiving irradiation at 0.54 J/cm^2 also differed significantly ($P \leq 0.0205$) from the 1.45 J/cm^2 treatment group at days 14, 16, 18, 21 and 23. Thus these findings demonstrate a further delay in wound closure in the 1.45 J/cm^2 treatment group.

In summary, prior irradiation with X-ray doses of 20 Gy caused a significant delay in the rate of wound healing by day 7 when compared to the non-X-ray irradiated group. Treatment with MLI at 0.18 J/cm^2 and 0.54 J/cm^2 had no effect upon the rate of wound closure in this model when compared to the X-ray irradiated Control group. However, treatment with MLI at 1.45 J/cm^2 caused a highly significant delay in the rate of wound healing by day 14 when compared to the other experimental groups.

DISCUSSION

The purpose of the current study was to determine the dose-dependence effect of MLI upon the rate of wound closure in a radiation impaired wound in murine skin. Results demonstrated that prior irradiation with X-ray doses of 20 Gy caused a significant delay in the rate of wound healing by day 7 when compared to the non-X-ray irradiated Control group, thus supporting previous findings [41]. MLI at 0.18 and 0.54 J/cm^2 had no effect upon the rate of wound closure when compared to the X-ray irradiated Control; however, monochromatic light irradiation at 1.45 J/cm^2 caused a significant delay in the rate of wound healing by day 14 when compared to the other experimental groups.

The normal wound healing response is a continuum of events divided into three overlapping stages. First, the initial inflammatory stage dominates the first few days of the wound healing process, and this is a period of active cellular migration. Between days 2 and 3 postwounding, the second stage begins. This proliferative stage lasts for 3 weeks and is marked by the production of collagen, wound contraction by myofibroblasts, and neovascularisation. The remodelling stage is the final stage of wound healing, beginning ~3 weeks postwounding, and lasting up to 2 years. During this time the tissue slowly regains the properties of normal skin [42,43]. In contrast, radiation-impaired wound healing is characterised by metaplastic and proliferative changes in the parenchymal cells that may lead to dermal atrophy, contraction, and susceptibility to necrosis, together with a reduction in wound strength [40]. Inui et al [44] have reported that whereas X-ray radiation reduces the rate of wound healing, its effect was dose dependent. It has previously been reported that X-ray radiation using a single dose of between 18 Gy and 20 Gy delivered by surface irradiation to the skin prior to wounding causes a significant wound healing deficit [38,45]. Indeed, prior irradiation using 20 Gy has been shown to cause a delay of 7 days in the time taken for the wound to shrink to 20% of that on day 0 [41]. Such radiation kills cells by impairment of their reproductive integrity; therefore, proliferating fibroblasts would be lost early, which could ultimately interfere with wound contraction and the production of primary collagen in the scar [40]. Thus, surface irradiation of the skin results in slower healing of open wounds and provides an in vivo system for the evaluation of topical dressings or

procedures designed to promote wound healing [38].

The impaired wound healing model used in the current study demonstrated an extended proliferative phase in the healing process, whereas at the inflammatory and tissue remodelling stages, this delay was not significant. Kumar and Jagestia [46] proposed that in this type of wound, there may be a delayed progression of the epidermal cells through the cell cycle, which may in turn lead to reduced fibroblastic function in the granulation bed. Results from the current study showed that the effect of X-ray irradiation was short-lived, delaying wound healing only during the proliferative stage, between days 7 and 18. Although such a delay allows time to evaluate the effects of various therapeutic procedures [38,41], it is incorrect to assume that a radiation-impaired model is directly comparable to pathological wounds found clinically, and, therefore, results of these studies may have only limited relevance to clinical situations [8,18]. This notwithstanding, it has previously been reported by a number of authors that laser irradiation stimulates the proliferative phase of wound healing [6,8,19,47]. In contrast, other authors have demonstrated effects during the inflammatory phase [48]. The current study has demonstrated no such stimulatory effect.

One proposed mechanism of action of photobiostimulation is the absorption of light energy by the mitochondria, which increases cell energy and cell membrane permeability and stimulates the release of the chemical mediators involved in wound repair [8,47,49,50]. Given the lack of such a stimulatory effect, it would appear that this mechanism did not occur at the parameters investigated in the current study. These findings are thus in agreement with Hall et al. [24], who also reported no stimulatory effect on the rate of wound closure of open skin wounds in rats using a pulsed GaAs (904 nm) laser at radiant exposures between 0.4 J/cm^2 and 4 J/cm^2. However, many investigators have reported that wound healing in animals and humans is accelerated following irradiation by a variety of low power lasers at doses of between 1–4 J/cm^2 [19,28,32,47,51].

Allendorf et al. [25] suggested that laser light penetration of tissue and eschar debridement are concerns with wound healing. Wounds left untouched, such as those in the current study, may not allow the maximum amount of light to reach the tissue. However, higher radiant exposures such as those used by Kana et al. [19] at 10 J/cm^2 and 20 J/cm^2 also have shown no stimulatory effects; therefore this explanation is unlikely. In addition, Allendorf et al. [25] proposed that anaesthesia produces a systemic effect that may retard or augment the wound healing process. They suggested that if the effects of anaesthesia are profound enough, they may obscure or overwhelm the effects of laser treatment.

Several investigators have reported isolated improvements in the normal healing process using laser therapy, but the beneficial effects do not appear to change the overall time required for complete wound repair [52]. One explanation proposed is that the healing process already proceeds at a near optimal rate in normal tissue; therefore, laser therapy will not increase this rate any further [52]. Karu [53] and Broadley et al. [22] supported this, suggesting that stimulation by light may take place only when growth rate is slow, e.g., in diabetic or age-compromised wound healing where the effects of such irradiation could be more obvious. Thus normal wounds that are likely to be repairing at an optimal level may show no response to laser therapy. In the current study, X-ray radiation was, therefore, used to impair the wound healing process so that the effect of this modality could be better investigated. Other authors have used a variety of models of impaired wound healing in an effort to provide a more clinically relevant situation. Yu et al. [52] investigated the effects of an argon dye laser (630 nm) on diabetic mice. Findings of this study demonstrated a significant increase in the rate of wound closure following irradiation using a radiant exposure of 5 J/cm^2 when compared to controls. Histological evaluation also showed improved epithelialisation, cellular content, granulation tissue formation, and collagen deposition in the laser-treated group.

When searching for an improved experimental model more relevant to the clinical setting, mice may not be the optimal choice for such investigations. Because of their loose skin, wounds in mice heal predominantly by wound contraction rather than by epithelialisation, such as occurs in human skin [2]. Therefore, any conclusions made from studies on mice may not be directly relevant to humans and can be used only to guide further research and (perhaps) explore mechanism(s) of action. Due to its similarity to human skin, pig skin may represent a more suitable wound healing model for such investigations.

In addition to providing an alternative and more suitable wound healing model, it also may

be beneficial to examine wounds histologically in order to detect differences at the cellular level. Future work in this area should also involve characterisation of the wound-healing model following treatment with laser therapy by a variety of methods. Routine histology should be used to determine cell types, cellular proliferation, angiogenesis, and the inflammatory response, whereas immunohistochemistry can be used to identify activated cells, specific cell types, and cytokine production.

To summarise, results of the current study provide no evidence of the claimed stimulatory effects of MLI upon the rate of wound closure at these parameters and instead showed an inhibitory effect at the highest radiant exposure investigated (1.45 J/cm^2). Similar results were obtained by Kana et al. [19], who also showed a inhibition in the healing rate in rats following treatment using a higher radiant exposure of 20 J/cm^2.

The management of chronic ulceration/delayed wound healing represents a significant problem for a variety of health professionals. The elderly, those confined to bed, and long-term diabetics often present with sores and ulceration that defy conventional treatment and cause considerable discomfort and suffering for the patient. These wounds are rarely a cause of mortality, but often lead to significant deterioration in the quality of life and an enormous cost associated with hospitalization. Therefore, there is a need to understand the deficit in the repair process induced by such complications and to develop therapeutic strategies for intervention to help address the dysfunction in the repair process.

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